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Methods for Obtaining Plant Varieties

TECHNICAL FIELD

The present invention relates to nucleotide sequences which encode polypeptides involved in the DNA mismatch repair systems of plants, and to the polypeptides encoded by those nucleotide sequences. The invention also relates to nucleotide sequences and polypeptide sequences for use in altering the DNA mismatch repair system in plants. The invention also relates to a process for altering the DNA mismatch repair system of a plant cell, to a process for increasing genetic variations in plants and to processes for obtaining plants having a desired characteristic.

BACKGROUND OF THE INVENTION

Plant breeding essentially relies on and makes use of genetic variation which occurs naturally within and between members of a family, a genus, a species or a subspecies. Another source of genetic variation is the introduction of genes from other organisms which may or may not be related to the host plant.

Allelic loci or non-allelic genes which constitute or contribute to desired quantitative (e.g. growth performance, yield, etc.) or qualitative (e.g. deposition, content and composition of seed storage products; pathogen resistance genes; etc.) traits that are absent, incomplete or inefficient in a species or subspecies of interest are typically introduced by the plant breeder from other species or subspecies, or *de novo*. This introduction is often done by crossing, provided that the species to be crossed are sexually compatible. Other means of introducing genomes, individual chromosomes or genes into plant cells or plants are well known in the art. They include cell fusion, chemically aided transfection (Schocher et al., 1986, Biotechnology 4: 1093) and ballistic (McCabe et al., 1988, Biotechnology 6: 923), microinjection (Neuhaus et al., 1987, TAG 75: 30), electroporation of protoplasts (Chupeau et al., 1989, Biotechnology 7: 53) or microbial transformation methods such as Agrobacterium mediated transformation (Horsch et al., 1985, Science 227: 1229; Hiei et al., 1996, Biotechnology 14: 745).

However, when a foreign genome, chromosome or gene is introduced into a plant, it will often segregate in subsequent generations from the genome of the recipient plant or plant cell during mitotic and meiotic cell divisions and, in consequence, become lost from the host plant or plant cell into which it had been introduced. Occasionally, however, the introduced genome, chromosome or gene physically combines entirely or in part with the genome, chromosome or gene of the host plant or plant cell in a process which is called recombination.

Recombination involves the exchange of covalent linkages between DNA molecules in regions of identical or similar sequence. It is referred to here as homologous recombination if donor and recipient DNA are identical or nearly identical (at least 99%

base sequence identity), and as homeologous recombination if donor and recipient DNA are not identical but are similar (less than 99% base sequence identity).

The ability of two genomes, chromosomes or genes to recombine is known to depend largely on the evolutionary relation between them and thus on the degree of sequence similarity between the two DNA molecules. Whereas homologous recombination is frequently observed during mitosis and meiosis, homeologous recombination is rarely or never seen.

From a breeder's perspective, the limits within which homologous recombination occurs, therefore, define a genetic barrier between species, varieties or lines, in contrast to homeologous recombination which can break this barrier. Homeologous recombination is thus of great importance for plant breeding. Accordingly there is a need for a process for enhancing the frequency of homeologous recombination in plants. In particular, there is a need for a process of increasing homeologous recombination to significantly shorten the length of breeding programs by reducing the number of crosses required to obtain an otherwise rare recombination event.

At least in *Escherichia coli*, homologous and homeologous recombination are known to share a common pathway that requires among others the proteins RecA, RecB, RecC, RecD and makes use of the SOS induced RuvA and RuvB, respectively. It has been suggested that mating induced recombination follows the Double-Strand Break Repair model (Szostak et al., 1983, Cell 33, 25-35), which is widely used to describe genetic recombination in eukaryotes. Following the alignment of homologous or homeologous DNA double helices the RecA protein mediates an exchange of a single DNA strand from the donor helix to the aligned recipient DNA helix. The incoming strand screens the recipient helix for sequence complementarity, seeking to form a heteroduplex by hydrogen bonding the complementary strand. The displaced homologous or homeologous strand of the recipient helix is guided into the donor helix where it base pairs with its counterpart strand to form a second heteroduplex. The resulting branch point then migrates along the aligned chromosomes thereby elongating and thus stabilising the initial heteroduplexes. Single stranded gaps (if present) are closed by DNA synthesis. The strand cross overs (Holliday junction) are eventually resolved enzymatically to yield the recombination products.

Although in wild type *E. coli* homologous and homeologous recombination are thus mechanistically similar if not identical, homologous recombination in conjugational crosses *E. coli* x *E. coli* occurs five orders of magnitude more frequently than homeologous recombination in conjugational crosses *E. coli* x *S. typhimurium* (Matic et al. 1995; Cell 80, 507-515). The imbalance in favour of homologous recombination was shown to be caused largely by the bacterial MisMatch Repair (MMR) system since its

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inactivation increased the frequency of homeologous recombination in *E. coli* up to 1000 fold (Rayssiguier et al. 1989, Nature 342, 396-401).

In *E. coli*, the MMR system (reviewed by Modrich 1991, Annual Rev Genetics 25, 229-253) is composed of only three proteins known as MutS, MutL and MutH. MutS recognizes and binds to base pair mismatches. MutL then forms a stable complex with mismatch bound MutS. This protein complex now activates the MutH intrinsic single stranded endonuclease which nicks the strand containing the misplaced base and thereby prepares the template for DNA repair enzymes.

During recombination, MMR components inhibit homeologous recombination. In vitro experiments demonstrated that MutS in complex with MutL binds to mismatches at the recombination branch point and physically blocks RecA mediated strand exchange and heteroduplex formation (Worth et al., 1994; PNAS 91, 3238-3241). Interestingly, the SOS dependent RuvAB mediated branch migration is insensitive to MutS/MutL, explaining the observed slight increase in SOS dependent homeologous recombination. Homeologous mating even induces the SOS response, thereby taking advantage of RuvAB induction (Matic et al. 1995, Cell 80, 507-515).

The MMR system thus appears to be a genetic guardian over genome stability in *E. coli*. In this role it essentially determines the extent to which genetic isolation, that is, speciation, occurs. The diminished sensitivity of the SOS system to MMR, however, allows (within limits) for rapid genomic changes at times of stress, providing the means for fast adaptation to altered environmental conditions and thus contributing to intraspecies genetic variation and species evolution.

The important role of MMR in preserving genomic integrity has been established also in certain eukaryotes. In its efficiency, the human MMR, for example, may even counteract potential gene therapy tools such as triple-helix forming oligonucleotides including RNA-DNA hybrid molecules (Havre et al., 1993, J. Virology 67: 7234-7331; Wang et al., 1995, Mol. Cell. Biol. 15: 1759-1768; Kotani et al., 1996, Mol. Gen. Genetics 250: 626-634; Cole-Strauss et al., 1996, Science 273: 1387-1389). Such oligonucleotides are designed to introduce single base changes into selected DNA target sequences in order to inactivate for example cancer genes or to restore their normal function. The resulting base mismatches however are recognised by the mismatch repair system which then directs removal of the mismatched base, thereby reducing the efficiency of oligonucleotide induced site-specific mutagenesis.

To date, two families of related genes, homologous to the bacterial *MutS* and *MutL* genes have been identified or isolated in yeast and mammals (recent reviews by Arnheim and Shibata, 1997, Curr. Opinion Genet. Dev. 7, 364-370; Modrich and Lahue, 1996, Annual Rev. Biochem. 65, 101-133; Umar and Kunkel, 1996, Eur. J. Biochem. 238, 297-307). Biochemical and genetic analysis indicated that eukaryotic MutS homologs (MSH)

and MutL homologs (MLH, PMS), respectively, fulfil similar protein functions as their bacterial counterparts. Their relative abundance, however, could reflect different mismatch specificity and/or specialisation for different tissues or organelles or developmental processes such as mitotic versus meiotic recombination.

5 To date, six different genes homologous to *MutS* have been isolated in yeast (*yMSH*), and their homologs have been found in mouse (*mMSH*) and human (*hMSH*), respectively. Encoded proteins yMSH2, yMSH3 and yMSH6 appear to be the main MutS homologs involved in MMR during mitosis and meiosis in yeast, where the complementary proteins MSH3 and MSH6 alternatively associate with MSH2 to recognise
10 different mismatch substrates (Masischky et al., 1996, *Genes Dev.* 10, 407-420). Similar protein interactions have been demonstrated for the human homologs hMSH2, hMSH3 and hMSH6 (Acharya et al., 1996, *PNAS* 93, 13629-13634).

MutL homologs (MLH and PMS), recently reviewed by Modrich and Lahue (1996, *Annual Rev. Biochem.* 65, 101-133) have so far been found in yeast (yMLH1 and
15 yPMS1), mouse (mPMS2) and human (hMLH1, hPMS1 and hPMS2). The hPMS2 is a member of a family of at least 7 genes (Horii et al., 1994, *Biochem. Biophys. Res. Commun.* 204, 1257-1264) and its gene product is most closely related to yPMS1. Prolla et al. (1994, *Science* 265, 1091-1093) presented evidence for yPMS1 and yMLH1 to physically associate with each other and, together, to interact with the MutS homolog
20 yMSH2 to form a ternary complex involved in mismatch substrate binding.

However, while medical interest in mismatch repair has prompted extensive research on MMR in bacteria, yeast and mammals, MMR genes have not been isolated from higher plants prior to the present invention and no attempts to adjust the plant MMR to plant breeding needs have been reported.

25 SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided an isolated and purified DNA molecule comprising a polynucleotide sequence encoding a polypeptide functionally involved in the DNA mismatch repair system of a plant. In one form of this embodiment, the invention provides an isolated and purified DNA molecule comprising a
30 polynucleotide sequence encoding a polypeptide which is homologous to a mismatch repair polypeptide of a yeast or of a human. More particularly, the invention provides polynucleotide sequences encoding polypeptides which are homologous to the mismatch repair polypeptides MSH3 and MSH6 of *Saccharomyces cerevisiae*. Still more particularly, the invention provides the coding sequences of the genes *AtMSH3* and
35 *AtMSH6* of *Arabidopsis thaliana*, as defined hereinbelow, and polynucleotide sequences encoding polypeptides which are homologous to polypeptides encoded by *AtMSH3* and *AtMSH6*.

According to a second embodiment of the invention, there is provided an isolated and purified polypeptide functionally involved in the DNA mismatch repair system of a plant, for example a polypeptide which is homologous to a mismatch repair polypeptide of a yeast or of a human such as a polypeptide encoded by the genes *AtMSH3* or *AtMSH6* of *Arabidopsis thaliana*, as defined hereinbelow.

According to a third embodiment of the invention, there is provided an isolated and purified DNA molecule comprising a polynucleotide sequence selected from the group consisting of (i) a sequence encoding a polynucleotide which is capable of interfering with the expression of a plant polynucleotide sequence encoding a polypeptide which is homologous to a mismatch repair polypeptide of a yeast or of a human and thereby disabling said plant polynucleotide sequence; and (ii) a sequence encoding a polypeptide capable of disrupting the DNA mismatch repair system of a plant.

According to a fourth embodiment of the invention there is provided a chimeric gene comprising a DNA sequence selected from the group consisting of (i) a sequence encoding a polynucleotide which is capable of interfering with the expression of a plant polynucleotide sequence encoding a polypeptide which is homologous to a mismatch repair polypeptide of a yeast or of a human and thereby disabling said plant polynucleotide sequence, and (ii) a sequence encoding a polypeptide capable of disrupting the DNA mismatch repair system of a plant; together with at least one regulation element capable of functioning in a plant cell. Examples of such regulation elements include constitutive, inducible, tissue type specific and cell type specific promoters such as 35S, NOS, PR1a, AoPR1 and DMC1. Typically, a chimeric gene of the fourth embodiment will also include at least one terminator sequence, more typically exactly one terminator sequence.

In the third and fourth embodiments, said interference, by said polynucleotide sequence, with the expression of a plant polynucleotide sequence encoding a polypeptide which is homologous to a mismatch repair peptide of a yeast or a human typically occurs by hybridisation or by co-suppression.

According to a fifth embodiment of the invention there is provided a plasmid or vector comprising a chimeric gene of the fourth embodiment. A vector of the fifth embodiment may be, for example, a viral vector or a bacterial vector.

According to a sixth embodiment of the invention, there is provided a plant cell stably transformed, transfected or electroporated with a plasmid or vector of the fifth embodiment.

According to seventh embodiment of the invention, there is provided a plant comprising a cell of the sixth embodiment.

According to an eighth embodiment of the invention, there is provided a process for at least partially inactivating a DNA mismatch repair system of a plant cell, comprising

transforming or transfecting said plant cell with a DNA sequence of the third embodiment or a chimeric gene of the fourth embodiment or a plasmid or vector of the fifth embodiment, and causing said DNA sequence to express said polynucleotide or said polypeptide.

5 According to a ninth embodiment of the invention, there is provided a process for increasing genetic variation in a plant comprising obtaining a hybrid plant from a first plant and a second plant, or cells thereof, said first and second plants being genetically different; altering the mismatch repair system in said hybrid plant; permitting said hybrid plant to self-fertilise and produce offspring plants; and screening said offspring plants for
10 plants in which homeologous recombination has occurred. For example, homeologous recombination may be evidenced by new genetic linkage of a desired characteristic trait or of a gene which contributes to a desired characteristic trait.

According to a tenth embodiment of the invention there is provided a process for obtaining a plant having a desired characteristic, comprising altering the mismatch repair
15 system in a plant, cell or plurality of cells of a plant which does not have said desired characteristic, permitting mutations to persist in said cells to produce mutated plant cells, deriving plants from said mutated plant cells, and screening said plants for a plant having said desired characteristic.

In a preferred form of the ninth and tenth embodiments of the invention, the step of
20 altering the mismatch repair system comprises introducing into said hybrid plant, plant, cell or cells a chimeric gene of the fourth embodiment and permitting the chimeric gene to express a polynucleotide which is capable of interfering with the expression of a plant polynucleotide sequence in a mismatch repair gene of the hybrid plant, plant, cell or cells, or a polypeptide capable of disrupting the DNA mismatch repair system of the hybrid
25 plant or cells.

In other embodiments, the invention provides (a) an oligonucleotide capable of hybridising at 45°C under standard PCR conditions to a DNA molecule of the first embodiment; (b) an oligonucleotide capable of hybridising at 45°C under standard PCR conditions to the DNA of SEQ ID NO: 18 and (c) an oligonucleotide capable of
30 hybridising at 45°C under standard PCR conditions to the DNA of SEQ ID NO:30; with the proviso that the oligonucleotide of (a), (b) and (c) is other than SEQ ID NO:1 or SEQ ID NO:2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a diagrammatic representation of the primer sequences used to
35 isolate *AtMSH3*.

Figure 2 is a plasmid map of clone 52, showing restriction enzyme cleavage sites in the 5' half of the full-length cDNA for *AtMSH3*.

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Figure 3 is a plasmid map of clone 13, showing restriction enzyme cleavage sites in the 3' half of the full-length cDNA for *AtMSH3*.

Figure 4 is a sequence listing of the coding sequence of *AtMSH3*, together with a deduced sequence of the encoded polypeptide.

5 Figure 5 is a protein alignment of yeast (*Saccharomyces cerevisiae*) and *Arabidopsis thaliana* MSH3 protein.

Figure 6 provides a diagrammatic representation of the primer sequences used to isolate *AtMSH6*.

Figure 7 is a plasmid map of clone 43, showing restriction enzyme cleavage sites in 10 the 5' half of the full-length cDNA for *AtMSH6*.

Figure 8 is a plasmid map of clone 62, showing restriction enzyme cleavage sites in the 3' half of the full-length cDNA for *AtMSH6*.

Figure 9 is a sequence listing of the coding sequence of *AtMSH6*, together with a deduced sequence of the encoded polypeptide.

15 Figure 10 is a protein alignment of yeast (*Saccharomyces cerevisiae*) and *Arabidopsis thaliana* MSH6 protein.

Figure 11 is a genomic sequence listing of *AtMSH6*.

Figure 12 is a plasmid map of plasmid pPF13.

Figure 13 is a plasmid map of plasmid pPF14.

20 Figure 14 is a plasmid map of plasmid pCW186.

Figure 15 is a plasmid map of plasmid pCW187.

Figure 16 is a plasmid map of plasmid pPF66.

Figure 17 is a plasmid map of plasmid pPF57.

Figure 18 is a diagrammatic representation of an antisense gene construction for use 25 in homeologous meiotic recombination.

Figure 19 is a plasmid map of plasmid p3243.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the inventors' discovery that there exist in higher plants genes which are homologous to MMR genes in *E. coli*, and to MMR genes in 30 yeasts and humans.

Thus, the inventors have identified genes, herein designated *AtMSH3* and *AtMSH6*, of the plant *Arabidopsis thaliana* which encode the proteins AtMSH3 and AtMSH6. These plant proteins are homologous to yMSH3 and yMSH6, respectively. The present inventors have isolated cDNAs encoding the proteins AtMSH3 and AtMSH6 and have 35 isolated the complete gene encoding AtMSH6. Given the teaching herein, other genes (for example AtMSH2, and genes of other plants) may be obtained which are involved in DNA mismatch repair in plants, including other genes which encode polypeptides homologous to MMR proteins of yeasts or humans, such as genes which encode

polypeptides homologous to yeast MSH2, MLH1 or PMS2, or to human MLH1, PMS1 or PMS2. For example, given the teaching herein, genes of members of the *Brassicaceae* family or of other unrelated families, for example the *Poaceae*, the *Solanaceae*, the *Asteraceae*, the *Malvaceae*, the *Fabaceae*, the *Linaceae*, the *Canabinaceae*, the *Dauaceae* and the *Cucurbitaceae* family, and which encode polypeptides homologous to MMR proteins of yeasts or humans may be obtained.

Examples of plants whose genes encoding polypeptides homologous to MMR proteins of yeasts or humans may be obtained given the teaching herein include maize, wheat, oats, barley, rice, tomato, potato, tobacco, capsicum, sunflower, lettuce, artichoke, safflower, cotton, okra, beans of many kinds including soybean, peas, melon, squash, cucumber, oilseed rape, broccoli, cauliflower, cabbage, flax, hemp, hops and carrot.

Within the meaning of the present invention, a first polypeptide is defined as homologous to a second polypeptide if the amino acid sequence of the first polypeptide exhibits a similarity of at least 50% on the polypeptide level to the amino acid sequence of the second polypeptide.

A procedure which may be followed to obtain genes *AtMSH3* and *AtMSH6* is described in Example 1. Essentially the same technique may be applied to obtain other mismatch repair genes of *Arabidopsis thaliana*, and essentially the same technique as exemplified herein may be applied to cDNA obtained by reverse transcription of RNA from other plants. Alternatively, given the sequence information disclosed herein, other degenerate oligonucleotide primers, especially oligonucleotides of the invention which are capable of hybridising at 45°C under standard PCR conditions (such as the conditions described in Example 1 using primers UPMU and DOMU) to *AtMSH3* and/or *AtMSH6* may be designed and obtained for use in isolating sequences of plant mismatch repair genes which are homologous to *AtMSH3* or *AtMSH6*, from other plants. Similarly, oligonucleotides of the invention which are capable of hybridising at 45°C under standard PCR conditions to plant mismatch repair genes of plants other than *Arabidopsis thaliana* also fall within the scope of the present invention and may be utilised to obtain mismatch repair genes of still other plants. Typically, such oligonucleotides are capable of hybridising at 45°C under standard PCR conditions to a DNA molecule which encodes a polypeptide which is homologous to a mismatch repair polypeptide of a yeast or a human. The temperature at which oligonucleotides of the invention hybridise to *AtMSH3* and/or *AtMSH6*, or to plant mismatch repair genes of plants other than *Arabidopsis thaliana*, or to DNA molecules which encode polypeptides which are homologous to a mismatch repair polypeptide of a yeast or a human may be higher than 45°C, for example at least 50°C, or at least 55°C, or at least 60°C or as high as 65°C.

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The successful gene isolation disclosed herein demonstrates for the first time the existence of MMR in higher plants and indicates the presence of other plant MMR genes. For example, genes encoding the plant homologs of MSH1, MSH2, MSH4, MSH5, PMS1, PMS2 and MLH1 may be identified given the teaching herein. Such genes, as well as those specifically described herein, separately or in combination, are useful in manipulating the plant MMR for plant breeding purposes. Thus, for example, the plant MMR may be altered by including in a plant cell a polynucleotide sequence as defined herein above with reference to the third embodiment of the invention, and causing the polynucleotide sequence to express either a polynucleotide which disables a plant MMR gene, or a polypeptide which disrupts the plant's MMR system.

The DNA molecule of the third embodiment of the invention includes a polynucleotide sequence (herein referred to as a MMR altering gene) which may for example encode sense, antisense or ribozyme molecules characterised by sufficient base sequence similarity or complementarity to the gene to be altered to permit the antisense or ribozyme molecule to hybridise with the plant MMR gene in vivo or to permit the sense molecule to participate in co-suppression. Alternatively, the MMR altering gene may encode a protein or proteins which interfere with the activity of a plant MMR protein and thus disrupt the plant's MMR system. For example, such encoded proteins may be antibodies or other proteins capable of interfering with MMR protein function, such as by complexing with a protein functionally involved in plant MMR thereby disrupting the MMR of the plant. An example of such a protein is the MSH3 protein of *Arabidopsis thaliana* described herein or a protein of another plant which is homologous to the MSH3 protein of *A. thaliana*. For instance, overexpression of MSH3 in a plant cell causes MSH2 present in the cell to be substantially completely complexed, disrupting the mismatch repair mechanism or mechanisms in the cell which are functionally dependent on the presence of a complex of MSH2 with MSH6. Similarly, mismatch repair mechanisms which depend on the presence of a complex of MSH2 and MSH3 may be disrupted by the overexpression of MSH6.

A chimeric gene of the fourth embodiment, incorporating a MMR altering gene, may be prepared by methods which are known in the art. Similarly, the MMR altering gene may be introduced into a plant cell, regenerating tissue or whole plant by techniques known in the art as being suitable for plant transformation, or by crossing. Known transformation techniques include *Agrobacterium tumefaciens* or *A. rhizogenes* mediated gene transfer, ballistic and chemical methods, and electroporation of protoplasts.

The MMR altering gene or genes are typically expressed from suitable promoters. Suitable promoters may direct constitutive expression, such as the 35S or the NOS promoter. Usually, however, the promoter will direct either inducible or tissue specific (e.g. callus; embryonic tissue; etc.), cell type specific (e.g. protoplasts; meiocytes; etc.) or developmental (e.g. embryo) expression of the altering gene or genes, in order for the

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MMR system to function in tissue types or cell types, or at developmental stages of the plant, in which it is not desirable for the MMR system to be altered. Using such promoters, therefore, the activity of a MMR altering gene may be limited to a specific stage during plant development or it may be altered by controlling conditions external to the plant, and the deleterious effects of a permanently disabled or altered DNA mismatch repair system in a plant may be avoided. Examples of suitable promoters which are not constitutive are known in the art and include inducible promoters such as *PR1a* (reviewed by Gatz, 1997, Annual Rev. Plant Phys. Plant Mol. Biol. 48: 89), tissue specific promoters such as *AtPR1* (Sabahattin et al., 1993, Biotechnology 11: 218), and cell-type specific promoters such as *DMC1*.

A chimeric gene in accordance with the invention may further be physically linked to one or more selection markers such as genes which confer phenotypic traits such as herbicide resistance, antibiotic resistance or disease resistance, or which confer some other recognisable trait such as male sterility, male fertility, grain size, colour, growth rate, flowering time, ripening time, etc.

The process of the tenth embodiment of the invention provides, for example, a process for generating intraspecies genetic variation by altering the mismatch repair system in a plant cell, in regenerating plant tissue or in a whole plant. The plant cell, regenerating tissue or whole plant includes and expresses one or more MMR altering genes which are capable of altering mismatch repair in the plant cell, regenerating tissue or whole plant. Alteration of MMR may be achieved, for example, by inactivating the genes encoding plant MSH3 and/or plant MSH6. It is preferred to inactivate the plant MSH3 and MSH6 encoding genes at the same time and in the same plant cell, regenerating tissue or whole plant. Typically in this preferred form of the invention inactivation of either plant MSH3 or MSH6 alone is insufficient to substantially alter the plant's mismatch repair system and only when both MSH3 and MSH6 are inactivated simultaneously is the plant's mismatch repair system sufficiently altered to prevent the MMR system from recognising base pair mismatches, base insertions or deletions as a result of DNA replication errors, DNA damage, or oligonucleotide induced site-specific mutagenesis. However, in some applications of the invention, inactivation of only one gene may also be used to cause genomic instability or increase the efficiency of site-specific mutagenesis.

If desired, the MMR altering gene or genes may later be rendered non-functional or ineffective, or may be removed from the genome of the plant cell, regenerating tissue or whole plant in order to restore mismatch repair in the plant cell, regenerating tissue or whole plant. The MMR altering gene or genes may be inactivated by means of known gene inactivation tools, such as ribozymes, or may be removed from the genome using gene elimination systems known in the art, such as *CRE/LOX*. It is preferred to render two genes, whose gene products combine to incapacitate MMR, ineffective by separating

the altering genes through segregation. Therefore, in a preferred embodiment of the invention a first plant cell or plant is generated in which only plant *MSH3* is incapacitated, and a second plant cell or plant is generated in which only plant *MSH6* is incapacitated. The combination of both genomes, for example by crossing, then produces significant
5 MMR deficiency in those cells or plants which have inherited both altering genes. If the altering genes are expressed from unlinked loci, gene segregation restores MMR activity in the progeny of the cells or plants.

In a process of the ninth embodiment of this invention, homeologous recombination is enhanced between different genomes, chromosomes or genes in plant cells or plants by
10 altering MMR in said plant cells or plants. Such genomes, chromosomes or genes are characterised in that they originate from different plant families, genera, species, subspecies, plant varieties or lines. Hybrid plant cells or hybrid plants may be produced by crossing, by cell fusion or by other techniques known in the art. These plant cells or plants are further characterised by expressing one or more genes that are capable of
15 altering mismatch repair in the plant cell or plants.

In the process of the ninth embodiment, the homeologous recombination is typically for the purpose of introducing a desired characteristic in the hybrid plant. In this typical application of the process of the ninth embodiment, and in the process of the tenth embodiment the desired characteristic may be any characteristic which is of value to the
20 plant breeder. Examples of such characteristics are well known in the art and include altered composition or quality of leaf or seed derived storage products (e.g. oil, starch, protein), altered composition or quality of cell walls (e.g. decrease in lignin content), altered growth rate, prolonged flowering, increased plant yield or grain yield, altered plant morphology, resistance to pathogens, tolerance to or improved performance under
25 environmental stresses of various kinds, etc.

In a preferred form of the tenth embodiment, an MMR altering gene is co-introduced along with the homeologous genome, chromosome or gene of another plant cell or plant into an MMR proficient plant cell or MMR proficient plant to produce a hybrid plant cell or hybrid plant in which homeologous recombination can occur.
30 Suitably, the MMR proficient plant cell or MMR proficient plant may also include an MMR altering gene. For example a gene capable of inactivating plant *MSH3* may be co-introduced along with the homeologous genome, chromosome or gene of another plant cell or plant into an MMR proficient plant cell or MMR proficient plant in which *MSH6* is inactivated. A resultant hybrid plant in which homeologous recombination occurs will
35 include both the *MSH3* and *MSH6* altering genes and its MMR system will therefore be inactivated.

In this form of the invention, if hybrid plants are to be produced by crossing, the MMR altering gene preferably originates from the male parent, thus ensuring that the

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MMR altering gene is always introduced and is not present in the recipient cell. That is, the MMR of the recipient cell, prior to introduction of the MMR altering gene, is typically proficient. Alternatively, if an MMR altering gene is present in a recipient cell it may be ineffective or inefficient on its own, or it may be linked to an inducible or tissue specific or cell type specific promoter which only renders the MMR altering gene active under limited conditions.

Thus, in a preferred form of the process of the ninth embodiment, the MMR system of the hybrid plant is initially unaltered. In this form of the process, the step of altering the mismatch repair system may comprise introducing into the hybrid plant, or cells thereof, a MMR altering gene, such as by *Agrobacterium tumefaciens* or *A. rhizogenes* mediated gene transfer, ballistic and chemical methods, and electroporation of protoplasts.

The MMR altering gene or genes are typically expressed from suitable promoters, as described above. Preferably, the promoter is transcriptionally active in mitotically and meiotically active tissue and/or cells to ensure MMR alteration after chromosome pairing at mitosis and meiosis, respectively. The preferred timing for MMR alteration is at meiosis, because recombinant genomes, chromosomes or genes are directly transmitted to the progeny. A suitable meiocyte specific promoter is for example the *DMC1* promoter from *Arabidopsis thaliana* ssp. *Ler.* (Klimyuk and Jones, 1997, Plant J. 11, 1-14). However, mitotic homeologous recombination is also a desirable outcome as somatic recombination events can be transmitted to offspring due to the totipotency of plant cells and the lack of predetermined germ cells in plants.

If desired, the MMR altering gene or genes may later be rendered non-functional or ineffective, or may be removed from the hybrid plant or hybrid plant cells, in order to restore mismatch repair in the hybrid plant or hybrid plant cells. The MMR altering gene or genes may be inactivated by means of known gene inactivation tools as described herein above.

EXAMPLES

Example 1. Cloning of the *AtMSH3* and *AtMSH6* coding sequences

Isolation of partial *AtMSH3* and *AtMSH6* consensus sequences

Degenerate oligonucleotides UPMU (SEQ ID NO:1) and DOMU (SEQ ID NO:2)

UPMU CTGGATCCACIGGICCAA(C/T)ATG

DOMU CTGGATCC(A/G)TA(A/G)TGIGTI(A/G)C(A/G)AA

were used to isolate *AtMSH3* and *AtMSH6* sequences by PCR amplification.

Primers UPMU and DOMU correspond to conserved amino acid sequences of the proteins MutS (*E. coli* and *S. typhimurium*), HexA (*S. pneumoniae*), Rep1 (mouse) and Dcl1 (human). The conserved regions to which they are targeted are TGPNM for UPMU (amino acid positions 852-856 for *AtMSH6* and 816-820 for *AtMSH3*) FATHY or FVTHY

for DOMU (amino acid positions 964-968 for AtMSH6 and 928-932 for AtMSH3, respectively.) These primers have been used to isolate MSH2 and MSH1 from yeast (Reenan and Kolodner, Genetics 132: 963-973 (1992)) and MSH2 from *Xenopus* and mouse (Varlet et al., Nuc. Acids Res. 22:5723-5728 (1994)).

- 5 Template single strand cDNA was produced by reverse transcription of 2 µg total RNA from a cell suspension culture of *Arabidopsis thaliana* ecotype Columbia (Axelos et al. 1989, Mol. Gen. Genetics 219: 106-112). The PCR reaction was performed under the following conditions in a final volume of 100µl: 0.2mM dNTP, 1µM each primer, 1XPCR buffer, 1u *Taq* DNA polymerase (Appligene) in the presence of template cDNA. PCR
- 10 parameters were 5 minutes at 94°C, followed by 30 cycles of 40 seconds at 95°C, 90 seconds at 45°C, 1 minute at 72°C. The amplification products were cloned into pGEM-T vector (Promega) and sequenced. Two different clones were isolated, S5 (350bp) was homologous to *MSH3*, S8 (327bp) was homologous to *MSH6*. Complete cDNA sequences were then isolated according to the Marathon cDNA amplification kit procedure (Clontech).
- 15 In summary, this procedure involves producing double stranded cDNA by reverse transcription of 2µg polyA⁺ RNA from the cell suspension culture of *Arabidopsis*. Adaptors are ligated on each side of the cDNA. The ligated cDNA is used as a template for 5' and 3' RACE PCR reactions in the presence of primers that are specific for the adaptor on one side (AP1 and AP2), and specific for the targeted gene on the other side. A 5' and a 3'
- 20 fragment that overlap are thus produced for each gene. The complete gene coding sequence can be reconstituted taking advantage of a unique restriction site, if available, in the overlapping region. Specific details of this procedure as it was used to isolate *AtMSH3* and *AtMSH6* coding regions, are as follows.

Isolation of *AtMSH3* complete coding sequence

- 25 From the sequence of clone S5, primer 636 (SEQ ID NO:3) was designed:

636 TGCTAGTGCCTCTTGCAAGCTCAT.

Primer AP1 (SEQ ID NO:4) is complementary to a portion of an adaptor sequence which had been ligated to the 5' and 3' ends of *Arabidopsis* cDNA:

AP1 CCATCCTAATACGACTCACTATAGGGC.

- 30 PCR performed on the ligated cDNA with primers 636 and AP1 for the 5' RACE PCR was followed by a second round of amplification with the nested primers AP2 (SEQ ID NO:5) and S525 (SEQ ID NO:6)

AP2 ACTCACTATAGGGCTCGAGCGGC

S525 AGGTTCTGATTATGTGTGACGCTTTACTTA

- 35 (the latter was also designed to correspond to a part of the sequence of clone S5) and produced a 2720bp DNA fragment. Figure 1 provides a diagrammatic representation of the primer sequences used to isolate *AtMSH3*. Another primer (S51, SEQ ID NO:7)

S51 GGATCGGGTACTGGGTTTTGAGTGTGAGG

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was designed closer to the 5' border and permitted the determination of 99bp upstream to the ATG initiation codon. For the 3' RACE PCR, a first PCR reaction was performed with primers AP1 and 635 (SEQ ID NO:8).

635 GCACGTGCTTGATGGTGTTCAC

5 followed by a second round of amplification, using the nested primers AP2 and S523 (SEQ ID NO:9)

S523 TCAGACAGTATCCAGCATGGCAGAAGTA

which produced a DNA fragment of 890bp. Both DNA fragments were subcloned into pGEM-T and sequenced. Since PCR amplification using the Expand Long Template PCR 10 System (Boehringer-Mannheim) produced errors in the sequence, new oligonucleotides were designed to isolate those sequences again by PCR, but with the high fidelity DNA polymerase *Pfu*. PCR with primers 1S5 (SEQ ID NO:10) and S53 (SEQ ID NO:11)

1S5 ATCCCGGGATGGGCAAGCAAAAGCAGCAGACGA

S53 GACAAAGAGCGAAATGAGGCCCTTGG

15 amplified the 1244bp fragment clone 52 (SEQ ID NO:12, cloned into pUC18/*Sma*I). PCR with primers S52 (SEQ ID NO:13) and 2S5 (SEQ ID NO:14)

2S5 ATCCCGGGTCAAAATGAACAAGTTGGTTTGTAGTC

S52 GCCACATCTGACTGTTCAAGCCCTCGC

amplified the 2104bp clone 13 (SEQ ID NO:15, cloned into pUC18/*Sma*I). The complete 20 coding sequence of the *AtMSH3* gene was reconstructed in pUC18 by ligating the 5' half of *AtMSH3* (clone 52) to the 3' half of *AtMSH3* (clone 13) after digesting with *Bam*HI which has a unique cleavage site in the overlapping region of both clones. This manipulation yielded plasmid pPF26. The *Sma*I fragment from pPF26 contains the complete *AtMSH3* coding sequence. The remaining primers referred to in Figure 1 are as 25 follows:

S51 GGATCGGGTACTGGGTTTTGAGTGTGAGG (SEQ ID NO:16)

S525 AGGTTCTGATTATGTGTGACGCTTACTTA (SEQ ID NO:17)

Figures 2 and 3 provide plasmid maps of clones 52 and 13 respectively, showing restriction enzyme cleavage sites. The complete *AtMSH3* coding sequence (SEQ ID NO:18) 30 is 3246bp long and is shown in Figure 4 together with the deduced sequence (SEQ ID NO:19) of the encoded polypeptide. *AtMSH3* is clearly homologous to the yeast and mouse *MSH3* genes. A sequence alignment of polypeptides encoded by *AtMSH3* and that encoded by *Saccharomyces cerevisiae MSH3* is set out in Figure 5.

Isolation of the *AtMSH6* complete coding sequence and genomic sequences

35 The same procedure allowed isolation of the *AtMSH6* cDNA. Figure 6 provides a diagrammatic representation of the primer sequences used to isolate *AtMSH6*. For the 5' RACE PCR, primers 638 (SEQ ID NO:20) and AP1 (SEQ ID NO:4)

638 TCTCTACCAGGTGACGAAAAACCG

allowed the amplification of a 2889 DNA fragment. Primer S81 (SEQ ID NO:21)

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S81 CGTCGCCTTTAGCATCCCCCTTCCTTCAC

helped define the 142bp upstream to the ATG initiation codon. On the 3' side, RACE PCR was initially performed with primers S823 (SEQ ID NO:22) and AP1 (SEQ ID NO:4),

S823 GCTTGGCGCATCTAATAGAATCATGACAGG

5 and then with the nested primers 637 (SEQ ID NO:23) and AP2 (SEQ ID NO:5).

637 GACAGCGTCAGTTCTTCAGAATGC

to produce a 774bp DNA fragment. As for *AtMSH3*, those fragments were cloned and sequenced. Re-isolation of the DNA sequence using the high fidelity *Pfu* polymerase and newly designed primers 1S8 (SEQ ID NO:24) and S83 (SEQ ID NO:25) (for the 5' side) led 10 to a 2182 bp DNA fragment identified as clone 43 (SEQ ID NO:26, cloned in pUC18/SmaI), and a 1379bp clone identified as clone 62 (SEQ ID NO:27, also cloned in pUC18/SmaI).

1S8 ATCCCGGGATGCAGCGCCAGAGATCGATTTTGT

2S8 ATCCCGGGTTATTTGGGAACACAGTAAGAGGATT (SEQ ID NO:28)

S82 GCGTTCGATCATCAGCCTCTGTGTTGC (SEQ ID NO:29)

S83 CGCTATCTATGGCTGCTTCGAATTGAG

Figures 7 and 8 provide plasmid maps of clones 43 and 62 respectively, showing restriction enzyme cleavage sites. Clones 43 and 62 were digested by the *XmnI* restriction enzyme for 20 which a unique site is present in their overlapping region and then ligated. The complete *AtMSH6* coding sequence (SEQ ID NO:30) is 3330bp long and is shown in Figure 9 together with the deduced sequence (SEQ ID NO:31) of the encoded polypeptide. *AtMSH6* is clearly homologous to the yeast and mouse *MSH6* genes. A sequence alignment of polypeptides encoded by *AtMSH6* and that encoded by *Saccharomyces cerevisiae MSH6* is 25 set out in Figure 10.

An *AtMSH6* genomic sequence was also isolated from a genomic DNA library constituted after partial *Sau3A*I digestion of DNA from the *Arabidopsis* cell suspension. 8062bp were sequenced that covered the *AtMSH6* gene and show colinearity with the cDNA. 16 introns are found scattered along the gene. The complete genomic sequence 30 (SEQ ID NO:98) is shown in Figure 11.

Example 2. A measure of somatic variation in MMR deficient plants

Constructs

Constructs with antisense *AtMSH3* or antisense *AtMSH6* or both *AtMSH3/AtMSH6* under the control of a single 35S promoter have been inserted into the binary vector 35 pPZP121 (Hajdukiewicz et al., Plant Mol. Biol. 23, 793-799) between the right and left borders of the T-DNA. The pPZP121 plasmid confers chloramphenicol resistance to *Escherichia coli* or *Agrobacterium tumefaciens* bacteria. The *aacC1* gene is carried by the T-DNA and allows selection of transformed plant cells on gentamycin (Hajdukiewicz et al., Plant Mol. Biol. 25, 989-994). For the purpose of expressing antisense constructs, a 35S

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promoter/terminator cassette with a polylinker was introduced into pPZP121. The 3' ends of the considered genes have been chosen since this region seems more efficient for antisense inhibition. For *AtMSH3* this corresponds to clone 13 (2104bp), for *AtMSH6* this corresponds to clone 62 (1379bp). Clone 13 comprises 2104bp of the 3' region that were cut off the pUC18 vector by *Sal*I/*Sst*I restriction, blunted with T4 DNA polymerase and ligated into the T4 DNA polymerase blunted *Bam*HI site of pPZP121/35S, creating clone pPF13. The same procedure was followed for the 3' region of *AtMSH6* clone 62 (1379bp) thus creating plasmid pPF14. For the double constructs, the 3' region (from clone 62) of *AtMSH6* was introduced ahead of the *AtMSH3* region into pPF13 creating pCW186 and reciprocally, the 3' region of *AtMSH3* (from clone 13) was introduced ahead of *AtMSH6* into pPF14, creating pCW187.

These constructs were introduced into the Arabidopsis cells (as described below) of wildtype Columbia and of the Columbia tester line.

An alternative strategy to antisense inhibition of *AtMSH6* comes from experiments of Marra et al. (1998, Proc. Natl. Acad. Sci USA 95, 8568-8573) who show that overexpression of functional *MSH3* results in depletion of *MSH6* protein in human cells. This depletion may generate a mismatch repair mutant phenotype.

For the purpose of overexpressing functional *AtMSH3* protein in plant cells, the complete *MSH3* coding region was excised from pPF26 (example 1) by digestion with *Sma*I, and was inserted into the *Sma*I site of pCW164. The resulting construct was named pPF66. It contains a complete *AtMSH3* gene under the control of the 35S promoter inside the left (LB) and right (RB) border of the T-DNA. This T-DNA also contains the *hpt2* gene for gentamycin selection. Plasmid pPF66 was introduced into Arabidopsis cells as described below. One cell clone was selected which clearly overexpressed the *AtMSH3* gene as shown by Northern analysis. Figures 12-16 provide plasmid maps of plasmids pPF13, pPF14, pCW186, pCW187 and pPF66, respectively.

Construction of tester construct

For the purpose of Forward Mutagenesis Assays, a tester construct was built containing the coding regions for *nptII*, *codA*, *uidA*. All three genes are driven by the 35S promoter and are terminated by the 35S terminator. This construct was obtained by introducing an *Eco*RI fragment encoding the *codA* cassette (2.5kb) and a *Hind*III fragment encoding the *uidA* (*GUS*) cassette (2.4kb) into the pPZP111 vector (Hajdukiewicz et al., 1994, Plant Mol Biol 23: 793-799) which already contained the *nptII* expression cassette. This new plasmid was named pPF57. *NptII* is used to select for transformed plant cells. *GUS* is used to analyse the degree of gene silencing in the construct (i.e. to identify cell lines in which the transgenes are expressed), and *codA* is used as a marker for forward mutagenesis (described below).

The plasmid map of pPF57 is provided in Figure 17.

Plant cell transformation

The constructs are introduced into *Agrobacterium* by electroporation. Plant cells are then transformed by co-cultivation. A suspension culture of *Arabidopsis thaliana* cells that has been established by Axelos et al. (1992, Plant Physiol. Biochem. 30, 1-6) may be used. One day old freshly subcultured cells are diluted five times into AT medium (Gamborg B5 medium, 30g/l sucrose, 200µg/l NAA). 10µl of saturated *Agrobacterium* containing the transforming T-DNA constructs are added to 10ml diluted cells in a 100ml erlenmeyer. The co-cultivation is agitated slowly (80rpm) for 2 days. The cells are then washed 3 times into AT medium and finally resuspended in the same initial volume (10ml). The culture is agitated for 3 days to allow expression before plating on selection plates (AT/BactoAgar 8g/l+gentamycin 50µg/ml). Transformed individual calli are isolated 3 weeks later.

Tester Strain

The tester construct on plasmid pPF57 was introduced into *Arabidopsis* cells of wildtype Columbia using the transformation protocol described above. Among 10 candidate transformants, one cell clone was shown (by Southern analysis) to have a unique T-DNA insertion. All three genes were shown to be functional in this cell line as indicated by resistance to kanamycin, blue staining in the presence of X-Glu (*GUS*), and sensitivity to 5-fluoro-cytosine (*codA*).

MMR altering genes (described above) were then introduced individually into the tester line and transformed cells are used for analysis of both Microsatellite Instability and Forward Mutagenesis.

Microsatellite analysis

Microsatellites have been described in *Arabidopsis* (Bell and Ecker, 1994, Genomics 19, 137-144). The present Example is based on a study of instability of microsatellites that are polymorphic amongst different ecotypes. DNA is extracted from the transformed calli. Specific primers have been defined that are used to amplify the microsatellite sequence. One of the two primers is previously P³² labelled by T4 kinase. In case of a polymorphic variation, new PCR products appear that do not follow the expected pattern of migration on a polyacrylamide gel. This is a commonly observed feature for MMR deficient cells in yeast or mammalian cells.

In particular, the present Example describes a study on microsatellites ca72 (CA₁₈), ngal72 (GA₂₉), and ATHGENEA(A₃₉), chosen because they belong to the types predominantly affected in human mismatch repair deficient tumors. The size of these microsatellites is not conserved from one *Arabidopsis* ecotype to the other.

Arabidopsis cells which are transformed with an MMR altering gene (above) and control cells not expressing the MMR altering gene are allowed to form calli. DNA is

rapidly extracted from the calli and is analysed for microsatellite instability as described in detail by Bell and Ecker 1994, Genomics 19, 137-144. In summary, the relevant microsatellite is amplified by PCR using P32 labelled primers. The PCR products are separated on a DNA sequencing gel for size determination. Size differences between
 5 microsatellites from transformed and control cells not expressing the MMR altering gene in question indicate microsatellite instability as a result of MMR alteration.

The sequences of primers used for PCR amplification of microsatellites ca72 and nga172 are included in Table 1. PCR amplification of microsatellite ATHGENEA made use of a forward primer containing the sequence

10 ACCATGCATAGCTTAAACTTCTTG (SEQ ID NO:32)

and of a reverse primer containing the sequence

ACATAACCACAAATAGGGGTGC (SEQ ID NO:33).

The amplification for microsatellite ca72 revealed in Columbia control cells (with respect to the MMR altering gene) a 248 bp long PCR fragment instead of the published
 15 length of 124 bp. DNA sequencing verified this fragment as a CA₁₈ microsatellite.

Forward mutagenesis assay

Tester cells transformed with antisense *AtMSH3* or antisense *AtMSH6* or both *AtMSH3/AtMSH6* are analysed for the stability of the *codA* gene. The functional *codA* gene confers to sensitivity to 5-fluoro-cytosine (5FC), whereas a gene inactivating mutation in
 20 *codA* will confer resistance to 5FC. The frequency of resistant cells is therefore a good indicator of somatic variation as a direct result of MMR alteration. Variants resistant to 5FC are first analysed for GUS activity. If GUS is inactive, 5FC resistance is assumed to be due to gene silencing (all three genes are under the 35S promoter). If GUS is active, 5FC resistance is assumed to be due to forward mutations that have inactivated *codA*. PCR is
 25 then performed on the putative *codA* mutant genes which is then sequenced to confirm the presence of forward mutations in *codA*.

Besides *codA*, other marker genes may also be used for the Forward Mutagenesis Assay such as the *ALS* gene (conferring sensitivity to valine or to sulfonylurea; Hervieu and Vaucheret, 1996, Mol. Gen. Genet. 251 220-224; Mazur et al. 1987, Plant Physiol. 85 1110-
 30 1117).

Example 3. Homeologous meiotic recombination in *Arabidopsis thaliana*

A. Construction of a meiocyte specific gene expression cassette comprising the *DMC1* promoter and the *NOS* terminator

(i) The *DMC1* promoter may be used as published by Klimyuk and Jones, 1997,
 35 Plant J. 11.1-14). To obtain a more convenient alternative for gene cloning, a 3.3 Kb

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long subfragment of the *DMC1* promoter was obtained by PCR from genomic DNA of *Arabidopsis thaliana* (ssp. Landsberg erecta "Ler").

The PCR was done in three rounds:

Round One: A 3.7 Kb long product was obtained using the forward primer
5 DMCIN-A comprising the sequence

GAAGCGATATTGTTCGTG (SEQ ID NO:34)

and the reverse primer DMCIN-B comprising the sequence

AGATTGCGAGAACATTCC (SEQ ID NO:35).

The weak amplification product was then used as template for round two and three.

10 Round Two: A 3.1 Kb long product comprising the promoter and the 5' untranslated leader was obtained using forward primer DMCIN-1, which contained the sequence

acgcgtcgacTCAGCTATGAGATTACTCGTG (SEQ ID NO:36)

and introduced a *SalI* cloning site at the 5' end of the promoter fragment, and reverse
15 primer DMCIN-2 which contained the sequence

gctctagaTTTCTCGCTCTAAGACTCTCT (SEQ ID NO:37)

and introduced a *XbaI* site at the 3' end of the PCR fragment.

Round Three: A 0.2 Kb long product comprising the first exon/intron of the *DMC1* promoter was obtained using forward primer DMCIN-3, which contained the sequence

20 gctctagaGCTTCTCTTAAGTAAGTGATTGAT (SEQ ID NO:38)

and introduced a *XbaI* site at the 5' end of the PCR fragment, and reverse primer DMCIN-4, containing the sequence

tccccgggctcgagagatcctcatggTTTCTTCAGCTCTATGAATCC (SEQ ID NO:39)

and introduced at the 3' end of the PCR product restriction sites for *NcoI*, *BglII*, *XhoI* and
25 *SmaI*.

The products obtained in round Two and Three were digested with *XbaI* and subsequently ligated to reconstitute a 3.3 Kb long *DMC1* promoter from which the first two in-frame ATG start codons were replaced with a unique restriction site for *XbaI*. This promoter can be cloned between the restriction sites for *SalI* and *SmaI* of p3264,
30 which contains the *SacI-EcoRI* NOS terminator in pBIN19, to yield the entire expression cassette in pBIN19. This cassette contains the following cloning sites: *NcoI*, *BglII*, *XhoI*, *SmaI* and (already present on p3264) *KpnI* and *SacI*.

(ii) Another strategy yielded the following convenient *DMC1* promoter. A 1.8 kb DNA fragment comprising the 3' terminal part of the meiocyte specific *DMC1* promoter
35 was isolated by PCR from purified genomic DNA of *Arabidopsis thaliana* (ssp. Landsberg erecta "Ler"). The forward PCR primer (DMC1a) contained the sequence

acgcgtcgacGAATTCGCAAGTGGGG (SEQ ID NO:40)

and introduced a *SalI* cloning site at the 5' end of the promoter fragment. The reverse PCR primer (DMC1b) contained the sequence

ttccatggagatctcccgggtacCGATTGCTTCGAGGG (SEQ ID NO:41)

introducing a polylinker region at the 3' end of the promoter fragment. The PCR reaction was carried out with VENT DNA Polymerase (NEB) over 25 cycles using the following cycling protocol: 1 minute at 94°C, 1 minute at 54°C, 2 minutes at 72°C.

5 The PCR reaction yielded a blunt ended DNA fragment which was digested with restriction enzyme *SaII* and was cloned into the cleavage sites of restriction enzymes *SaII* and *SmaI* in plasmid p2030, a pUC118 derivative containing the *SacI-EcoRI* NOS terminator fragment of pBIN121. The cloning yielded plasmid p2031, containing the *DMC1* promoter-polylinker-NOS terminator expression cassette depicted in Figure 18.

10 B. Construction of an *MSH3* antisense gene under the control of the *DMC1* promoter

A 2.1 kb DNA fragment encoding the carboxyterminal part of AtMSH3 was removed from the polylinker of clone 13 described in Example 1 after (i) digestion with *Kpn*I, (ii) blunting of the DNA ends generated by *Kpn*I and (iii) digestion with *Bam*HI. The isolated fragment was then cloned in antisense orientation downstream of the *DMC1* promoter in plasmid p2031, which had been digested with restriction enzymes *Sma*I and *Bgl*II. This cloning yielded plasmid p2033 (Figure 18).

After digestion of p2033 with *EcoRI*, a 4.1 kb DNA fragment was recovered comprising the *DMC1* promoter, the partial *MSH3* cDNA in antisense orientation with respect to the promoter and the *NOS* terminator. This fragment was cloned into the *EcoRI* 20 restriction site of plant transformation vector pNOS-Hyg-SCV to yield plasmid p3242 (Figure 18).

C. Construction of a combined *MSH6/MSH3* antisense gene under the control of a single *DMC1* promoter

A 3.1 kb fragment, encoding in antisense orientation the partial *AtMSH6* and *AtMSH3* sequences and the 35S terminator, was isolated from pCW186 by digestion with *KpnI*. The fragment was treated with *Klenow* enzyme to blunt both ends. It was then cloned into the *SmaI* site of plasmid p3243 (a pNOS-Hyg-SCV derivative, illustrated in Figure 19), which had been opened to delete the region between the *SmaI* sites. Clones containing the fragment in the antisense orientation with respect to the *DMC1* promoter (described in A(ii) above) were identified by diagnostic digestion with *BamHI*. The selected construct was named p3261.

Another practical way of cloning the double antisense gene is as follows. A 1 kb DNA fragment encoding the carboxyterminal part of AtMSH6 is isolated from clone 62 described in Example 1 after digestion of clone 62 plasmid DNA with *Bam*HI, which
35 cleaves in the 5' polylinker region flanking the partial cDNA, and with *Eco*RI, which cleaves within the cDNA. The isolated fragment is treated with *Klenow* enzyme to blunt both its ends and is cloned into the recipient plasmid p2033 or p3242. For the purpose of

cloning, the recipient plasmid may be cleaved with either *Ava*I or *Nco*I and can be blunted with *Klenow* enzyme to produce blunt acceptor ends for fragment cloning. This cloning yields two opposite orientations of cloned fragment DNA with respect to the *DMC1* promoter. These can be identified by diagnostic digestion with restriction enzymes *Sca*I or *Xmn*I in conjunction with *Sac*I. The selected construct contains the *DMC1* promoter, the combined partial cDNAs for *AtMSH3* and *AtMSH6* (both cloned in antisense orientation with respect to the *DMC1* promoter) and the *NOS* terminator. If the recipient plasmid is p2033, the combined antisense gene under control the single *DMC1* promoter is recovered from the vector after *Eco*RI digestion and is cloned into the *Eco*RI restriction site of pNOS-Hyg-SCV.

D. Construction of a full-length *MSH3* sense gene under control of the *DMC1* promoter for overexpression of functional *MSH3* protein

Overexpression of *MSH3* protein was shown in human cells (Marra et al., 1998, Proc. Natl. Acad. Sci. USA 95, 8568-8573) to complex all available *MSH2* protein. This leaves *MSH6* protein without its partner, leading to the degradation of *MSH6* protein and eventually to a mismatch repair phenotype.

This phenomenon is exploited to increase homeologous meiotic recombination in Arabidopsis as an alternative to antisense inhibition of *AtMSH6*. For this purpose the full-length cDNA encoding *AtMSH3* is isolated from plasmid pPF66 by digestion with *Sma*I and is cloned into the *Sma*I site of the *DMC1* expression cassettes described in A(i).

E. Selection of Recombination markers on homeologous chromosomes of *Arabidopsis thaliana* subspecies *Landsberg erecta* (Ler), *Columbia* (Col) and *C24*, respectively

E(i). Visual recombination markers in *Arabidopsis th.* subspecies *C24*:

Agrobacterium mediated transformation with a T-DNA containing a *35S-GUS* gene, inactivated by insertion of a *35S-Ac* transposable element (Finnegan et al., 1993, Plant Mol. Biol. 22, 625-633), had yielded a *C24* line in which the T-DNA construct was integrated into chromosome 2. Genetic and molecular analysis of this line shows that the *Ac* transposon had excised from its T-DNA locus thereby restoring *GUS* activity, but had re-inserted into the chromosome at a distance of 16.4 cM, where it stayed fixed (due to disablement of *Ac*) within the *chlorina* gene. Insertional inactivation of the *chlorina* gene caused a bleached phenotype in those plants that were homozygous for this mutation. Because of the two linked phenotypic markers, *chlorina3A:Ac* and *GUS*, this *C24* line was used in crosses to wildtype Ler for analysis of meiotic homeologous recombination on chromosome 2 in conjunction with molecular recombination markers.

E(ii). Visual recombination markers in *Arabidopsis th.* *Ler*:

The Ler line NW1 (obtained from NASC, Nottingham, UK) contains one recessive visual marker per chromosome. i.e. *an-1* on Chr.1, *py-1* on Chr.2, *gll-1* on Chr.3, *cer2-1*

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on Chr.4. and *msl-1* on Chr.5. This line is used in crosses to wildtype C24 which expresses an MMR altering gene for analysis of meiotic homeologous recombination on chromosomes 1-5 in conjunction with molecular recombination markers listed in Table 1.

Other *Ler* lines from NASC have several visual markers in close proximity to each other on the same chromosome. When these lines are used for hybrid production, analysis of homeologous meiotic recombination may make use entirely of visual recombination markers. Particularly suitable for crossing to C24 wildtype that is expressing a MMR altering gene are the following *Ler* lines:

NW22: relative markers are *dis1* - (4 cM) - *ga4* - (11 cM) - *th1* on chromosome 1.

10 NW10: relevant markers are *tz-201* - (5 cM) - *cer3* on chromosome 5.

NW134, relevant markers are *ttg* - (4 cM) - *ga3* on chromosome 5.

NW24 (*abi3-1*) and NW64 (*gll-1*). When present in the same plant on chromosome 3, *abi3-1* and *gll-1* are 13 cM apart. Since this marker combination is not available from NASC, we have combined these markers by crossing line NW24 to line NW64. The F1 offspring were allowed to self-fertilise and to produce F2 seeds. F2 Plants which carry both markers as homozygous traits on the same chromosome can be identified firstly, by germinating F2 seeds on germination medium containing selective concentrations of abscisic acid, and subsequently, by identifying among the abscisic acid resistant plants those individuals which show the glabra phenotype.

20 E(iii) Molecular recombination markers in *Col*, *Ler* and C24:

The genome of *Arabidopsis thaliana* is interspersed with unique base sequences arranged as simple tandem repeats. Allelic repeats can vary in length between different *Arabidopsis* subspecies and when amplified by PCR yield diagnostic DNA products of different length named Simple Sequence Length Polymorphisms (SSLPs). Many SSLPs have been genetically mapped and have been assigned to unique chromosome locations on the recombinant inbred map (Bell and Ecker, 1994, Genomics 19, 137-144; Lister and Deans lines, Weeds World 4i, May 1997).

In Table 1 are listed 28 mapped and established SSLPs between *Ler* and *Col*. A number of PCR primer pairs are described herein (SEQ ID NO:42 to SEQ ID NO:97) which also yielded SSLPs between C24 and *Ler* (19 SSLPs) or between C24 and *Col* (25 SSLPs), respectively. Polymorphic SSLPs can be used as molecular markers in the analysis of homeologous recombination between genomes from these subspecies.

The PCR reactions (25 µL) were carried out over 35 cycles (15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C), with 0.25 U Taq DNA polymerase and 0.6 µg genomic DNA in reaction buffer containing 2 mM MgCl₂. PCR products were separated by agarose gel electrophoresis (4% ultra high resolution agarose) and visualised by ethidiumbromide staining. The results from the PCR experiments are summarised in

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Table 1, which also shows the sequence of PCR primers, primer annealing temperature (T_m), PCR product length and chromosome location of SSLP (with respect to the RI map of May 1997, Weeds World 4i).

F. Production of hybrid plants

- 5 C24 plants heterozygous for *chlorina3A:Ac/GUS* are crossed as male to emasculated wildtype *Ler* to produce *Ler/C24(chlorina3A, GUS)* hybrid seeds.

Due to the heterozygosity of the C24 parent, only 50 % of hybrid plants have inherited the *chlorina3A:Ac/GUS* locus. The remaining 50% of hybrid plants are wildtype with respect to *chlorina3A:Ac/GUS*. Since the mutant locus is linked to a kanamycin
10 resistance gene (contained on the same T-DNA as *GUS*) mutant plants can be pre-selected by germinating hybrid seeds on germination medium containing 50 mg/L kanamycin.

Ler plants homozygous for the five chromosome markers are male sterile (*ms1-1*) and are crossed without emasculation to wildtype C24 to produce *Ler(an-1, py-1, gl1-1, cer2-1, ms1-1)/C24* hybrid seeds.

- 15 Other *Ler* plants, which are male fertile, are crossed after emasculation of the female parent to produce *Ler/C24* hybrid seeds.

G. Introduction of *MSH3* and *MSH6/3* antisense genes into *Arabidopsis* and analysis of meiotic homeologous recombination

(i) Transformation of hybrid plants and analysis of homeologous meiotic recombination

- 20 The plant transformation vectors comprising the antisense genes described in (B) and (C) above are introduced into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al., 1991, Bio/Technology 9, 963-967) by electroporation. Recombinant *Agrobacterium* clones are selected on LB medium containing 50 mg/L rifampicin and 100 mg/L carbenicillin. Selected clones are used to infect roots of *Arabidopsis* hybrid plants (described in (F)
25 above) using the root transformation protocol of Valvekens et al. (1988, PNAS 85, 5536-5540) except that shoot and root inducing media contain hygromycin (10 mg/L) instead of kanamycin.

- Plants regenerated from roots of hybrid plants are genetic clones of root donating plants and therefore are again genetic hybrids of two *Arabidopsis* subspecies described in
30 (F). However, in contrast to the root donating plants, the regenerated hybrid plants also contain the introduced transgene and the co-introduced hygromycin resistance gene with the latter allowing these plants to grow on hygromycin containing culture medium.

- Hygromycin resistant plants are then allowed to enter the reproductive phase and to produce gametes by meiotic divisions of microspore and megaspore mothercells. At
35 meiosis, the *DMC1* promoter is activated and can direct the expression of antisense genes described in (B) and (C) above, leading to decreased *MSH6* and/or *MSH3* gene

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expression. This in turn depletes the gamete mothercells of MSH6 and/or MSH3 protein, thus causing alteration of MMR during meiotic divisions and increasing the recombination frequency between homeologous chromosomes.

Transgenic plants are then allowed to self-fertilise and to produce seeds. These seeds (F2 seeds with respect to hybrid production), and the plants derived therefrom, carry the homeologous recombination events which can be identified by using the visual and molecular recombination markers described in (E) above.

In case of homeologous recombination between chromosomes of *Ler* and *C24(chlorina3A:Ac, GUS)*, the analysis concentrates on chromosome 2 by selecting plants showing the visual phenotypic marker *chlorina*. This marker thus serves as a reference point as it indicates that respective chromosomes 2 originate from *C24*. Other markers, such as *GUS* or molecular markers, on chromosome 2 may then be used to identify chromosomal regions which are derived from the *Ler* chromosome as a result of homeologous recombination. F2 plants of control transformants not expressing the antisense gene(s) can be analysed in parallel and the results can be used for comparison to homeologous recombination results obtained in antisense plants.

(ii) Transformation of C24 wildtype, hybrid plant production and analysis of homeologous meiotic recombination

Introduction of MMR altering genes into wildtype *C24* is done using the root transformation protocol as described in G(i) for transformation of hybrid plants. Transformed plants are selected by resistance to either 10 mg/L hygromycin (in case of transformation with T-DNA's derived from pNOS-Hyg-SCV) or to 50 mg/L kanamycin (in case of transformation with T-DNA's derived from pBIN19).

Transgenic plants are then allowed to self-fertilise and to produce seeds (T1 seeds). Segregation of the antibiotic resistance gene in the T1 population then indicates the number of transgene loci. Lines with a single transgene locus (indicated by a 3:1 ratio of resistant:sensitive plants) are selected and are bred to homozygosity. This is done by collecting selfed seeds (T2) from T1 plants and subsequent testing of at least four independent T2 seed populations for segregation of the antibiotic resistance gene. T2 populations which do not segregate the antibiotic resistance gene are assumed to be homozygous for both the resistance gene and the linked MMR altering gene.

C24 plants homozygous for the MMR altering gene are then crossed to *Ler* lines homozygous for recessive visual markers (see E(ii)) to produce *C24/Ler* hybrid plants as described in (F). These F1 hybrids are then allowed to enter the reproductive phase and to produce gametes by meiotic division of microspore and megaspore mothercells. At meiosis, the *DMC 1* promoter is activated and can direct the expression of antisense or sense genes described in (B), (C) and (D) above, leading to decreased *MSH6* and/or *MSH3* gene expression. This in turn depletes the gamete mothercells of *MSH6* and/or *MSH3*

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protein, thus causing alteration of MMR during meiotic divisions and increasing the recombination frequency between the homeologous chromosomes of *C24* and *Ler*. Recombination events are then scored in the F2 generation.

For recombination analysis, the hybrid plants are allowed to self-fertilise and to produce F2 seeds. F2 plants are then preselected for a first visual marker. Since this marker is recessive, its visual presence indicates homozygosity for *Ler* DNA at the relevant locus. Those F2 plants which show this first visual marker are then analysed for the presence or absence of a second visual marker which in the *Ler* parent is closely linked to the first marker. Absence of the second visual marker indicates recombination between the relevant *C24* and *Ler* chromosomes between the first and second marker. The frequency of recombination in transgenic hybrids is compared to the recombination frequency in control hybrids not expressing the MMR altering gene.

Examples of recombination analysis are the following.

The *Ler* line NW22(*dis1*, *ga4*, *th1*) is used for crosses to transformed *C24*.

F2 plants are preselected first for thiamine requirement (*th1*) and then are further analysed for re-appearance of wildtype height (loss of *ga4*) and/or re-appearance of normal trichomes (loss of *dis1*) as a result of recombination.

The *Ler* line NW10(*tz-201*, *cer3*) is used for crosses to transformed *C24*.

F2 plants are then preselected first for thiazole requirement (*tz*) and then are further analysed for re-appearance of normal, i.e. non-shiny stems (loss of *cer3*) as a result of recombination.

The *Ler* line NW134 (*itg*, *ga3*) is used for crosses to transformed *C24*. F2 plants are first preselected for dwarfish appearance (*ga3*) and are then analysed for re-appearance of trichomes (loss of *itg*) as a result of recombination.

Ler plants homozygous for *abi3-1* and *gll-1* are used for crosses to transformed *C24*. F2 plants are first preselected for their ability to germinate in the presence of abscisic acid and are then analysed for re-appearance of trichomes on the leaves (loss of *gll-1*) as a result of recombination.

In the case of homeologous recombination between transformed *C24* and the *Ler* line NW1 (*an-1*, *py-1*, *gll-1*, *cer2-1*, *msl-1*), recombination analysis is similar the one described above, except that the second marker is not a visual marker but has to be a molecular marker. This is because the *Ler* parent carries only one visual marker per chromosome.

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TABLE 1: SSLP Markers in *Arabidopsis thaliana* Subspecies

Chromosome	RI Map Position	PCR Primer Pair	Primer Sequence	T _m [°C]	length/COL	length/LER	length/C24
I	2.3	ATEAT1 F ATEAT1 R	GCCACTGCGTGAATGATATG CGAACAGCCAAACATTAATCCC	57.8 58.2	172	162	162
I	9.3	NGA63 F NGA63 R	AACCAAGGCACAGAAAGC ACCCAAGTGATGCCACC	57.3 59.6	111	89	120
I	40.1	NGA248 F NGA248 R	TACCGAACCAAAACACAAAGG TCTGTATCTCGGTGAATTCTCC	56.1 58.2	143	129	no amplific.
I	81.2	NGA128 F NGA128 R	GGTCTGTGATGTCGTAAGTCG ATCTTGAAACCTTTAGGGAGGG	60.1 58.2	180	190	no amplific.
I	81.2	NGA280 F NGA280 R	CTGATCTCACGGACAATAGTGC GGCTCCATAAAAAAGTCACC	60.1 57.8	105	85	85
I	111.4	NGA111 F NGA111 R	CTCCAGTTGGAAGCTAAAGGG TGTTTTTTAGGACAAAATGGCG	60 70	128	162	170
II	ca. 7.5	NGA168 F NGA168 R	CCTTCACATCCAAAACCCAC GCACATACCCACAACCCAGAA	57.8 57.8	213	217	208

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II	ca. 48	NGA1126L	CGCTACGCTTTTCGGTAAAG	57.8	191	199	196
		NGA1126R	GCACAGTCCAAAGTCACAACC	59.9			
II	62.2	NGA361L	AAAGAGATGAGAATTGGAC	51.7	114	120	114
		NGA361R	ACATATCAATATATTAAAGTAGC	49.5			
II	73	NGA168 F	TCGTCTACTGCACTGCCG	59.6	151	135	135
		NGA168 R	GAGGACATGTATAGGAGCCTCG	61.9			
II	ca. 77	AthBIO2 L	TGACCTCCTCTTCCATGGAG	59.9	141	209	139
		AthBIO2 R	TTAACAGAAACCCAAAGCTTTC	54.5			
II	ca. 83	AthUBIQUE L	AGGCAAAATGTCCATTTCATTG	54.1	146	148	148
		AthUBIQUE R	ACGACATGGCAGATTCTCC	57.8			
III	3.4	NGA172 F	AGCTGCTTCCTTATAGCGTCC	60	162	136	140
		NGA172 R	CATCCGAATGCCATTGTTTC	55.4			
III	12.8	NGA126 F	GAAAAACGGCTACTTTCGTGG	56.1	119	147	no amplific.
		NGA126 R	CAAGAGCAATATCAAGAGCAGC	58.2			
III	17.5	NGA162 F	CATGCAATTGTCATCTGAGG	55.8	107	89	no amplific.
		NGA162 R	CTCTGTCACTCTTTTCCCTCTGG	60.1			

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III	81.8	NGA6 F	TGGATTTCCTCCTCTCTCAC	56.1	143	123	143
		NGA6 R	ATGGAGAAGCTTACACTGATC	56.1			
IV	19.8	NGA12 F	AATGTTGTCTCCTCCCTCCTC	59.9	247	234	220
		NGA12 R	TGATGCTCTCTCTGAACAAGAGC	58.2			
IV	24.1	NGA8 F	GAGGGCAAAATCTTTATTGGG	56.1	154	198	190
		NGA8 R	TGGCTTTCGTTTATAAACATCC	54.5			
IV	102	NGA1107 L	GCGAAAAACAAAAAATCCA	50.2	150	140	140
		NGA1107 R	CGACGAATCGACAGAAATTAGG	58			
V	11.8	NGA225 F	GAAATCCAAATCCAGAGAGG	58	119	189	119
		NGA225 R	TCTCCCCACTAGTTTGTGTCC	60.1			
V	16.7	NGA249 F	TACCGTCAATTCATCGCC	55.4	125	115	115
		NGA249 R	GGATCCCTAACTGTAAAAATCCC	58.2			
V	19.9	CA72 F	AATCCCAGTAACCAACACACA	56.3	124	110	110
		CA72 R	CCCAGTCTAACCCAGACCAC	61.9			
V	20	NGA151 F	GTTTGGGAAGTTTGTCTGG	55.8	150	120	130
		NGA151 R	CAGTCTAAAAACGAGAGTATGATG	58.6			

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V	24	NGA106 F	GTTATGGAGTTCTAGGCACG	60.1	157	123	130
		NGA106 R	TGCCCAATTTGTCTCTC	55.8			
V	37.8	NGA139 F	AGAGCTACCAGATCCGATGG	59.9	174	132	132
		NGA139 R	GGTTTCGTTTCACTATCCAGG	55.8			
V	50	NGA76 F	GGAGAAAATGTCACTCTCCACC	60.1	231	> 250	300
		NGA76 R	AGGCATGGGAGACATTTACG	57.8			
V	61.1	ATHSO191 L	CTCCACCAATCATGCAAATG	55.8	148	156	146
		ATHSO191 R	TGATGTTGATGGAGATGGTCA	53.7			
V	81.7	NGA129 F	TCAGGAGGAACATAAGTGAGGG	60.1	177	179	172
		NGA129 R	CACACTGAAGATGGTCTTGAGG	60.1			